·Commentary·

Relationship between Epstein-Barr virus infection and nasopharyngeal carcinoma pathogenesis

Chin-Tarng Lin

[Abstract] Nasopharyngeal carcinoma (NPC) is one of the common cancers among Chinese living in South China, Taiwan, Singapore, and several other countries or regions in distinct areas. The etiological factors have not been clearly identified yet. So far, no major gene related with hereditary factor has been identified in NPC carcinogenesis; however, some environmental factors, such as consumption of salted fish and long-term exposure to sulfuric-acid vapor, have been tentatively linked to NPC induction, while research has proposed that there is a close association between Epstein-Barr virus (EBV) and NPC pathogenesis. To investigate the relationship between NPC and EBV, we have established ten NPC cell lines. After extensive investigation, we conclude that EBV may establish an infection only in nasopharyngeal neoplastic cells, not in metaplastic epithelial cells, through the IgA receptor (secretory component protein)-mediated endocytosis. Our observations indicate that EBV plays an important role in enhancement of NPC progression, but is involved in neither the initiation nor the promotion of NPC pathogenesis.
Key words: nasopharyngeal neoplasm/etiology, molecular pathogenesis, Epstein-Barr virus

Nasopharyngeal carcinoma (NPC) is defined as a malignant tumor of the squamous metaplastic epithelia lining the surface of the nasopharynx,[1] which is normally lined with simple ciliated columnar epithelial cells and goblet cells (Fig. 1).[2] When the mucosal cells suffer from environment stimulation, the lining epithelia may show squamous metaplasia (Fig. 1).

According to the World Health Organization classification,[3] NPC can be divided into three groups: keratinizing squamous cell carcinoma (WHO type I), non-keratinizing carcinoma (WHO type II), and undifferentiated carcinoma (WHO type III). NPC has a remarkably distinctive ethnic and geographic distribution. It is endemic in Southeast Asia, North Africa, and Alaska. The annual incidence is about 26 per 100 000 persons in the endemic areas and less than 1 per 100 000 persons in Caucasian people from Western countries. Among the three histological types, undifferentiated carcinoma (type Ⅲ) is the most frequent in endemic areas; however, keratinizing squamous cell carcinoma (type Ⅰ) is more common in Europe and has a worse prognosis. In 2002, about 80 000 incident cases of NPC were diagnosed worldwide and the estimated number of deaths exceeded 50 000, making it the 23rd most common cancer in the world.[4-6] A high incidence has been reported among people of Guangdong Province and the Guangxi region of China, where the incidence of NPC reaches 50 or more per 100 000 people per year.[5] In Taiwan, it is the 14th most common cancer. However, among relatively young adults (25–45 years old), NPC is the fourth leading cancer-related cause of death,

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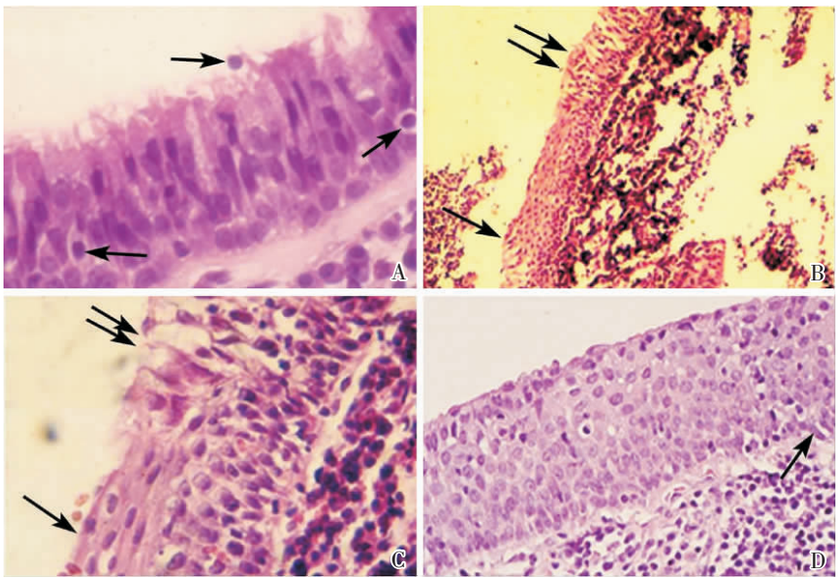

Figure 1 Histopathologic demonstration of normal nasopharyngeal mucosa, squamous metaplasia and nasopharyngeal carcinoma (NPC) in situ

A: Normal ciliated columnar epithelia of the nasopharynx infiltrated with a few lymphocytes (arrows) (HE ×100).

B. Focal squamous metaplasia of nasopharyngeal mucosa (between single and double arrows) (HE ×25).

C. Higher magnification from B (double arrows) showing the junction of squamous metaplasia (single arrow) and ciliated columnar epithelia (double arrows) (HE ×100).

D. Nasopharyngeal carcinoma in situ with early invasion (arrow) (HE ×50).

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having a percentage rank similar to the ranks of hepatoma, colon cancer, and esophageal cancer (Fig. 2) (Department of Health of Taiwan, 2005).

Research has linked three general risk factors to NPC etiology: genetic susceptibility, the environment, and Epstein-Barr virus (EBV) infection. Based on familial studies in Greenland[7] and identical-twins studies in Taiwan and other studies,[8-10] it was suggested that genetic background could be a factor related to NPC tumorigenesis. But more recent statistical studies on data concerning 1 903 Cantonese subjects revealed that the observed data were best explained by a multifactorial mode of inheritance for NPC. There was no evidence supporting the hypothesis that any major histocompatibility locus gene is involved in NPC tumorigenesis.[11]

Among the environmental risk factors of NPC is the consumption of preserved foods, in particular, Chinese salted fish, which has been associated consistently with an increased risk of NPC.[12-15] Long-term exposure to relatively low concentrations of sulfuric-acid vapors may also be associated with the development of NPC.[16-19] Regarding other factors, long-term cigarette smoking is associated with NPC risk but low levels of exposure to second-hand cigarette smoke and to alcohol consumption are not associated with disease risk.[20,21]

EBV infection was long thought to be the most important factor associated with NPC.[22-30] Indeed, research has proposed that EBV is perhaps the main etiological factor for NPC formation. This proposal rests on clinical observation and some basic studies that detected three interesting findings: (1) the high titer of the IgA antibody anti-EBV-VCA (viral capsid antigen) in the majority of NPC patients, (2) the presence of the EBV genome or gene products in the majority of NPC tissues, and (3) the monoclonality

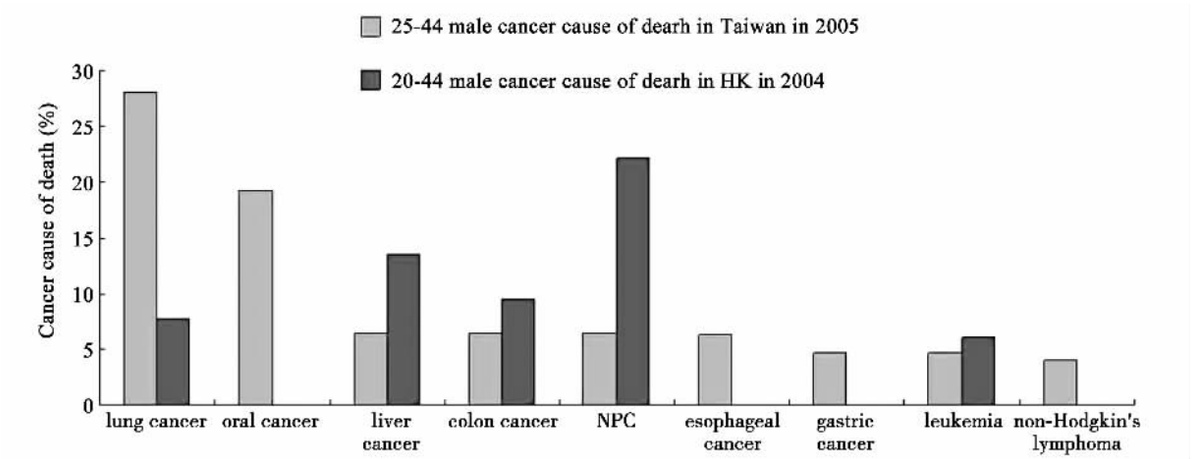

Figure 2 Comparison of the percentages of cancer causes of death in Taiwan and Hong Kong in young patients

of the EBV terminal repeat sequence identified in EBV-positive (EBV^+) NPC biopsy specimens. All of these studies seem to put too much emphasis on those cases in which EBV was detected.

However, a more detailed study[31] of nine established NPC cell lines showed that not all NPC cell lines contained EBV and that, for EBV^+ cell lines, only a small fraction of tumor cells contained EBV. The same results were also obtained from a study of original NPC biopsy specimens. In addition, those untransformed squamous metaplastic epithelial cells and mild dysplasia of the nasopharynx did not contain EBV signals examined by EBV-encoded RNA1 (EBER-1) in situ hybridization.[31] Other published studies in the literature have reported consistent findings.[32,33] These findings, as a whole, strongly suggest that EBV infection could be a secondary effect during NPC progression, but not the primary etiological factor. The following five facts further support this idea: (1) tumor cells from NPC cell lines and NPC biopsy specimens do not express the EBV receptor; (2) some tumor cells in NPC cell lines and in NPC biopsy specimens, but not in untransformed metaplastic epithelial cells and mild dysplastic cells, express secretory component (SC) proteins (IgA receptors);[34] (3) EBV infection can be obtained through IgA receptor-mediated endocytosis in NPC cell lines; (4) the monoclonality of the EBV terminal repeat sequence can be found in in vitro and in vivo EBV-infected NPC cells;[35,36] and (5) EBV-infected NPC cell lines show increasing production of epidermal growth factor receptor (EGFR), tumor growth factor-β (TGF-β), interleukin-1α (IL-1α), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF),[37] and other cell proliferation-related genes. These findings indicate the possibility that EBV infection can enhance the growth rate of NPC tumor cells and that EBV infection may very well play a role in the secondary effect during NPC progression rather than as a primary NPC etiological factor.

The following section discusses the detailed relationship between EBV and NPC.

1 Biological response of NPC cell lines to EBV infection

1.1 EBV signals in NPC cell lines

When NPC biopsy specimens are subjected to primary culture, certain NPC tumor fragments from certain patients can attach to the substratum of the Petri dish and start to proliferate and become the primary cultures. After several subcultures, a few primary culture cells proliferate continuously and become a cell line, while many other primary cultures stop growing and become differentiated and degenerated.[38] Using this method, we obtained ten NPC cell lines from 209 biopsy cases.[39] Southern blot analysis (SBA) revealed that no surviving cell lines showed the EBV DNA signal. But use of a polymerase chain reaction (PCR) plus SBA revealed that, although certain cell lines still exhibited no EBV signal, other cell lines exhibited EBV signals. If all lines have been subcultured for more than 30 passages, all lines become EBV-negative (EBV^-).[31] These phenomena suggest that only a fraction of NPC cells in the EBV^+ line are infected by EBV and that the EBV genome in the infected cells is of an episomal form.[31] No NPC cell line would contain the episomal EBV genome after long-term subculture,[31,40-44] unless the NPC cell line would contain an integrated EBV genome,[45-48] which is unusual and has not been seen in NPC biopsy specimens.

1.2 EBV infection pathway and exocytosis of EBV particles from infected cells

NPC cells cannot be infected by EBV if isolated EBV particles are co-cultured directly with EBV^- NPC cells. When we checked CD21 protein (EBV receptor) on NPC cell surface by immunostaining, no reaction product of anti-CD21 was detectable, indicating that NPC cells do not express CD21, so EBV can not directly infect NPC cells. This phenomenon postponed our experiment for several months. Therefore, we retraced our steps and reviewed the clinical manifestation in NPC patients, and finally we found that one of the most peculiar symptoms in most EBV-infected NPC patients is that a high titer of the IgA antibody anti-EBV-VCA was present in their sera (very few EBV-associated diseases may show moderate increase of this IgA antibody). We wonder whether IgA anti-EBV-VCA may play some roles in the EBV infection pathway. Therefore, we started to review the literature about the IgA transportation from the source of the plasma-cell secretion to the oral cavity.[49] According to our findings, the 'j' chain of IgA (secreted from plasma cells that infiltrate the mesenchymal tissue surrounding the salivary glands) can bind to the

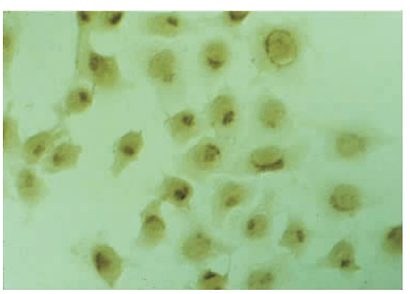

Figure 3 Localization of secretory component (SC) protein in NPC culture cells by immunohistochemistry (IHC ×200)

The reaction product of anti-SC is seen in the cytoplasm and on some plasma membranes of many tumor cells in the NPC-TW04 line. Some cells reveal immunostaining in the Golgi apparatus region.

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secretory component (SC) protein (we have referred to the SC protein as an IgA receptor) on the basolateral plasma membrane of salivary glandular epithelia. Then, IgA molecules are endocytosed. After transcytosis and excytosis, IgA molecules can finally be discharged into the lumen of the salivary gland and transported into the oral cavity.[49] Therefore, we used an immunolocalization method to observe whether our NPC cell lines also expressed the SC protein (IgA receptor). It was a surprise that most NPC cell lines expressed the SC protein on membrane and in cytoplasm. But in each NPC cell line, only a fraction of NPC cells, ranging from 45% to 95% of the total cell population of each of the nine NPC lines,[34] expressed the SC protein (Fig. 3). So, to treat NPC cells, we first used NPC patient serum with a high titer (1:640) of IgA anti-EBV-VCA (the IgA titer in the NPC patient's serum was between 1:40 and 1:640) as the source of the IgA antibody. Then, we incubated the NPC cells followed by

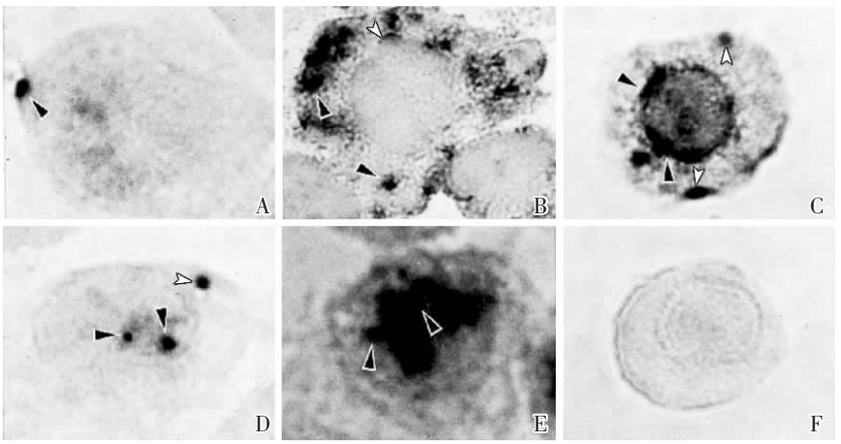

Figure 4 Montage of Epstein-Barr virus (EBV) infection pathway in NPC cells by in situ PCR hybridization (×390)

NPC-TW 01 cells were infected by EBV and IgA anti-EBV and incubated for 2–24 h. The EBV DNA signals are identified by in situ PCR hybridization. A to C: Incubation for 2 h. D and E: Incubation for 24 h. In the first 2 hours, the granular reaction product of the EBV DNA sequence is shown attached to the plasma membrane (A, arrowhead), in the cytoplasm (B, black arrowheads), at the subplasmal membrane region (C, white arrowheads), and the nuclear envelope (B, white arrowhead, and C, black arrowheads). After 24 hours, an occasional granular reaction product is still seen in the cytoplasm (D, white arrowhead), but most of the granular reaction products are shown in the nucleus (D, black arrowheads, and E, arrowheads). F: A negative control NPC cell, blocked by excess antibodies against SC and then incubated with EBV and IgA, shows no specific reaction product.

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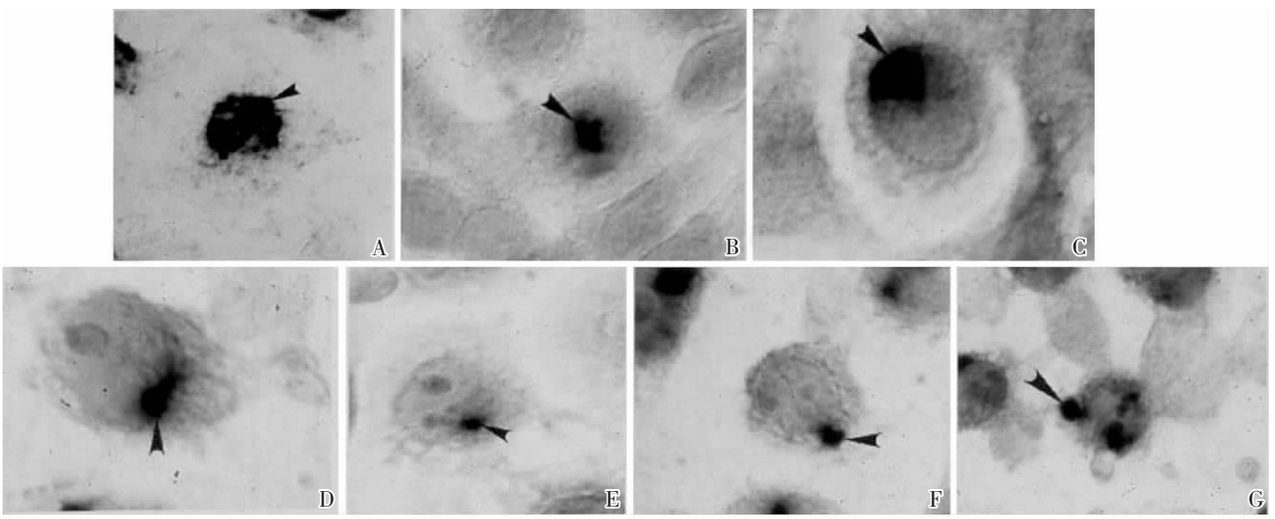

Figure 5 A montage of individual NPC cells infected by EBV by in situ PCR hybridization at high magnification

A: infected for 2 days; B–G: infected for 21 days. A: Many granular reaction products of EBV DNA are aggregated in the nucleus (arrowhead). B: A few granules are clustered in the nucleus (arrowhead). The reaction product of EBV signal is attached to the inner surface of the nuclear envelope (arrowhead) in C, overriding on the nuclear envelope (arrowhead) in D, in the cytoplasm (arrowhead) in E, and attached to the inner surface of the plasma membrane (arrowhead) in F. One of the granular reaction products is exocytosed and attached to the external surface of the cell membrane (arrowhead) in G. (A, B, G: ×200; C–F: ×350).

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incubation with EBV particles isolated from the B95-8 cell line. Using this "IgA receptor"-mediated endocytosis method to infect NPC cells, one can obtain high infection efficiency.[34] The EBV entry into NPC cells can be visualized by in situ PCR hybridization. The overall EBV entry pathway can be concluded that the EBV particles bind on the NPC cell membrane 1 h after incubation, and are present in host cytoplasm 2 h after infection; EBV signals gradually move toward the nuclear envelope, and are finally stabilized in the nuclei 24 h after infection (Fig. 4).[34] This infection model can retain the episomal viral genome in host cells for only 14 days.[37] After 2 weeks, a few EBV genomes start to move toward the inner nuclear membrane from the nucleoplasm, into the cytoplasm, and then close to the inner plasma membrane, and finally, the virus is exocytosed from a majority of the infected cell population at about day 28.[37] The whole process of exocytosis of EBV signals is verifiable by in situ PCR hybridization of EBV DNA and immunohistochemical staining of EBNA-1 in the EBV-infected NPC cells in a cell culture (Fig. 5).[37] EBV signals are no longer detectable 72 days after the start of the infection.[34] However, EBV$^+$ NPC cells remain identifiable in NPC xenografts in serial passages up to 50 times in SCID mice (our unpublished data). It is possible that some unidentified factors appearing in the in vivo microenvironment not only help preserve the EBV episome in the xenografts but also are absent in the cell culture medium. We believe that this infection method may occur in the tumor cells in NPC patients because it is nearly a normal physiological infection pathway. During our development of this infection method, a similar EBV-infection method for a colon cancer cell line was reported.[50] Other non-physiological methods for EBV infection of NPC cells include direct contact of EBV-releasing B lymphocytes incubated with IgG molecules[46] and CR21 plasmid-transfected NPC cells.[47] But the infection efficiency is rather low, and the EBV genome is also rarely retained in the infected cells after serial passages, unless the EBV genome becomes an integrated form in the infected cells using the selection marker.[48]

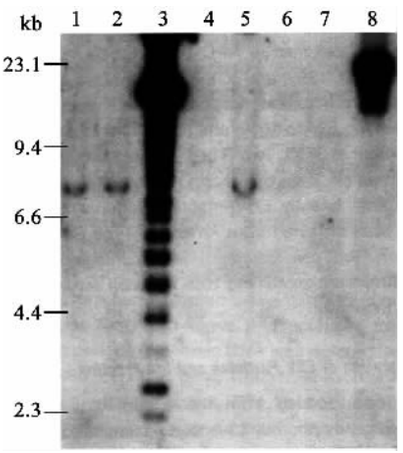

Figure 6 Southern blot analysis of EBV termini in polyclonal EBV-infected NPC cell lines

EBV particles were isolated either from the monoclonal B95-8 subline (EBVm) or from the original polyclonal B95-8 line (EBVp). They were used to infect NPC culture cells by the method of IgA-mediated endocytosis. All cellular DNA were hybridized with xho1 1.9-kb probe. Lanes 1 and 2: NPC-TW01 cell line at 60th passage, infected by monoclonal EBV particles, and incubated for 32 or 48 days; lane 3: B95-8 cell line, EBV particles isolated from the original polyclonal B95-8 cell line (the DNA was diluted at 1:100); lanes 4 and 7: uninfected NPC-TW04-30 and NPC-TW01-60 cell lines; land 5: NPC-TW04-30 cell line was infected by EBV particles and incubated for 40 days; lane 6: NHB, normal blood cells; lane 8: Raji cell line. A single homogenous band of 7.5 kb was found in both the clonal and polyclonal EBV-infected NPC cell lines (lanes 1, 2, and 5). The original B95-8 line in lane 3 shows multiple bands.

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1.3 Monoclonality versus polyclonality of the EBV genomic sequence in NPC cell lines and xenografts

Southern blot analysis of EBV DNA from NPC biopsy specimens demonstrates a monoclonality of the resident viral genome.[28,35] Previously, Raab-Traub and Flynn[28] had used this finding to claim that EBV infection takes place before the clonal expansion of a population of malignant cells. However, in vitro, NPC cells infected by EBV through the "IgA receptor"-mediated endocytosis method also demonstrate the monoclonality of the infected EBV terminal repeat sequence (Fig. 6).[35] The fact that most of the NPC cells in the in vitro infection (using 1×10^5 cells) are infected at the same time, and the EBV genomes extracted from those infected cells still show the monoclonality, but not the polyclonality, of the EBV terminal repeat (TR) sequence, indicates that the resident EBV genome in infected

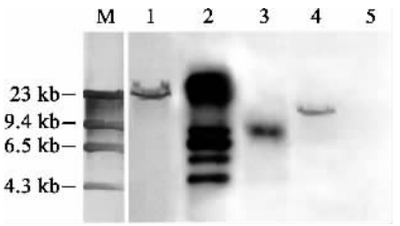

Figure 7 Southern blot analysis of EBV terminal repeat pattern in EBV$^+$ NPC

Lane M: marker; lane 1: Raji cell line; lane 2: B95-8 cell line; lane 3: in vivo EBV$^+$ NPC-TW01 tumor; lane 4: in vivo EBV$^+$ NPC-TW06 tumor; lane 5: an EBV$^+$ solid tumor produced by injection of NPC-TW01 cells. Except for lane 2, which has one major 18- to 30-kb band (probably derived from fused termini bands) and other multiple bands, lanes 1, 3, and 4 each has a homogeneous single band of terminal repeat EBV DNA sequences of different sizes. Lane 5 shows no EBV genome.

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epithelial carcinoma cells is different from the resident EBV genome in B cell infection. This finding is also substantiated by an in vivo xenograft experiment, in which SCID mice are injected with EBV$^-$ NPC cells at first; after a tumor nodule is formed, it is injected with IgA anti EBV-VCA and EBV-free particles separately at the same site. The produced xenografts also show a monoclonal EBV TR sequence, and the size of the TR sequence is different in different cell line-induced xenografts (Fig. 7).[35,36]

Therefore, the finding that monoclonality of EBV TR sequence in an NPC biopsy specimen cannot rigorously support the statement that EBV infection takes place before the clonal expansion of the total population of NPC cells. One possible explanation of this phenomenon is that during EBV infection, the infected host cells may contain a polyclonal TR sequence of the EBV genome, but after internal selection in NPC cells, only one TR sequence of the EBV genome can survive and stay in a patient's NPC cells.

1.4 Functional analysis of EBV in infected NPC cells

EBV infection in NPC cells can enhance mildly to moderately the proliferation, and migration and invasion ability of host cells. The degree of enhancement is cell line-dependent.[35,36] This effect may be due to the alteration of host gene expression regulated by EBV genomes. For example, the expression of invasion-related protease genes, such as matrix metalloproteinase-2 (MMP-2) and MMP-9 and heparanase, can be gradually up-regulated by 2.5 folds after 3 weeks of infection.[36] But the angiogenic factors, such as vascular endothelial growth factor (VEGF), is up-regulated by about 2.3 folds, while basic fibroblast growth factor (bFGF) is up-regulated by less than 1.5 folds at the 3rd week.[37] Similarly, some oncogenes (such as EGFR and TGF-α) and cytokines (such as IL-1β, IL-6, and GM-CSF) can be up-regulated mildly, but other oncogenes (such as TGF-β) and other cytokines (such as IL-2, IL-3, IL-4, IL-5, and so on) are not clearly regulated.[37] The alteration of host gene expression relative to EBV infection is gene type-dependent, and also is dependent on the EBV retention time in host cells.[36] Although the invasion-related gene expression can be slightly to moderately up-regulated by EBV, other genes such as the IgA receptor gene (SC gene) can be down-regulated.[36] The down regulation of SC gene expression in EBV-infected NPC cells may prevent the second EBV infection. This phenomenon may explain the fact that the presence of an EBV copy in each infected cell is consistent for a long time in vitro and probably is also consistent in the in vivo condition. In the genome-wide comparative scanning of NPC transcriptomes, the EBV infection's overall gene alteration of NPC host genes has demonstrated that EBV infection has a mild-to-moderate intensifying effect on the signals in NPC progression-related gene expression involving proliferation, cell cycle progression, motility, and anti-apoptosis-related genes and other genes.[51]

1.5 Functional analysis of EBV in infected xenografts in SCID mice

Most EBV$^-$ NPC cell lines can induce xenograft formation in SCID mice if the tumor cells grow in an optimal condition with sufficient cells.[36,38,39] However, the growth rate of EBV$^-$ NPC xenograft is not very fast. Usually, it may take two months to obtain a xenograft with a diameter of 1.0–1.5 cm. If EBV$^+$ NPC cells are injected into SCID mice, they can induce xenograft formation with a faster growth rate and larger tumor size than the EBV$^-$ cell-induced xenografts (Fig. 8A-D).[36,52]

One can observe the clonal proliferation of EBV$^+$ NPC cells in the EBV$^-$ tumor mass in the xenograft if EBV$^-$ xenograft grows to a tumor size of about 1.0 cm in diameter and is injected with the IgA anti EBV-VCA and EBV particles separately.[36] EBV$^+$ cells grow as a clonal expansion at the junction between EBV$^-$ cells and EBV$^+$ cells,[36] a picture similar to the NPC biopsy specimen.[31] Double localization of the EBV genome and certain host proteins in EBV$^+$ xenografts demonstrate that EBV up-regulates host genes only in cells that express those genes (Fig. 8E), not in cells that do not express the genes (Fig. 8F).[36,53] This finding suggests that EBV infection enhances the progression of NPC tumor growth. But this observation in animal xenografts does not rule out a role for EBV infection in the induction and early promotion of NPC development. To verify the conclusion that EBV plays a role in enhancement of NPC progression but is not involved in the initiation and promotion states of NPC tumorigenesis, we need to examine the human biopsy specimens with carcinoma in situ (CIS) and with severe dysphasia. The biopsy specimen data are discussed in the following section. We should keep in mind that unidentified factors can also enhance NPC tumor growth, independent of the effects of EBV.

2 EBV in NPC biopsy specimens

2.1 Localization of EBV

EBV (DNA, mRNA, or its protein) has been localized in a majority (not 100%) of WHO type Ⅱ and Ⅲ carcinomas and in a certain percentage of type I NPC biopsy specimens.[32,53–55] No matter what technology is used to identify EBV in NPC specimens, there are some NPC cases, especially the WHO type I carcinomas, showing no EBV signal.[31,36,53,54] On average, in EBV$^+$ NPC biopsy specimens, about 72%–82% of tumor cells are infected

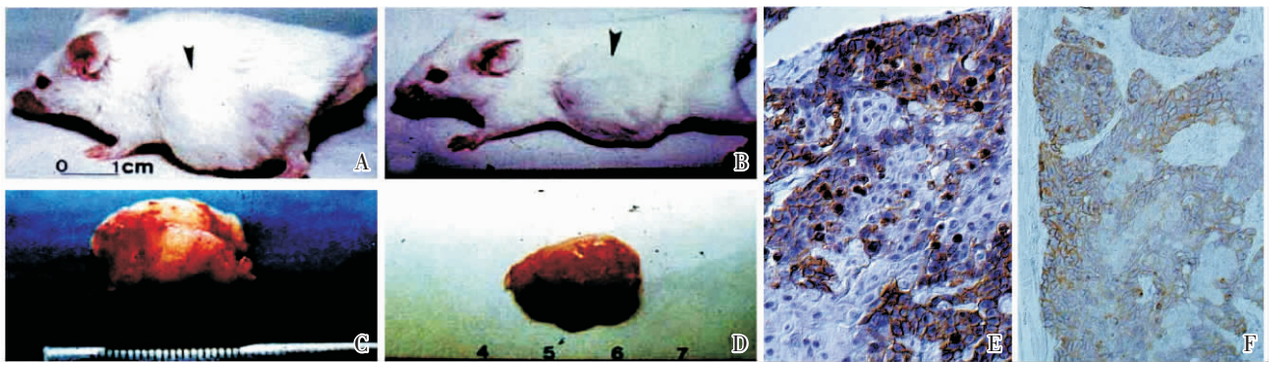

Figure 8 Identification of EBV signals in EBV$^+$ xenograft tumor tissue in SCID mice

EBV$^+$ NPC cells, 8 days after infection, were injected subcutaneously into the flank of SCID mice. After 4 weeks, the mice were killed and the tumor masses were measured. A, C: The gross morphology of the SCID mice bearing EBV$^+$ NPC cells and its xenograft tumor mass. B, D: The gross morphology of the SCID mouse bearing EBV$^-$ NPC cells and its xenograft tumor mass. The tumor mass in panel C is larger than that in panel D. E and F: Co-localization of EGFR and EBER-1 (EBER-1 in situ and IHC staining of EGFR ×50). E: Most tumor cells show clear EBER-1 signals (blue), but only a fraction also contains strong EGFR immunoreactivity (brown). F. No EBV signal is shown in the EBV- section, but EGFR immunoreactivity is seen in certain tumor cells; another group of tumor cells contain no EGFR reaction product.

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with EBV.[53] The number of EBV$^+$ cells in the biopsy specimens is variable from case to case. In some cases, 98% of NPC cells may be infected by EBV;[36] in other cases, less than 10% of NPC cells may be infected by EBV. Some NPC biopsy specimens exhibit a geographic distribution of EBV$^+$ cells with both clonal expansion and an invasive pattern in EBV$^-$ NPC nodules (Figs. 9 and 10).[31,34] This pattern can be seen in many WHO type Ⅰ and Ⅱ NPC specimens but in fewer of type Ⅲ specimens, especially the lymphoepithelioma-like NPC. However, many type III tumor tissues always exhibit some EBV$^-$ tumor cells (Figs. 9 and 10).[31,34,36,53,54]

2.2 Localization of IgA receptor (IgAR) protein in NPC biopsy specimens

SC protein can be seen in three places: (1) the epithelial cells of the salivary glands, (2) the ductal epithelia of the salivary glands, and (3) some ciliated columnar epithelia of normal nasopharyngeal mucosa (data not shown). But neither squamous metaplastic epithelia nor mild dysplastic cells of the nasopharynx express IgAR protein.[34] In most cases of CIS, including severe dysplasia and early invasive NPC, IgAR is not identified (Fig. 11A),[56] only certain sporadic tumor cells express IgAR (Figs. 11B-D and 12A-B)[34,56] However, in a few cases, IgAR$^+$ tumor cells can be seen in clusters (Fig. 11D). NPC cells in each subtype express IgAR in patches and clusters (Figs. 11D and 12B).[34,56] In fact, keratinizing

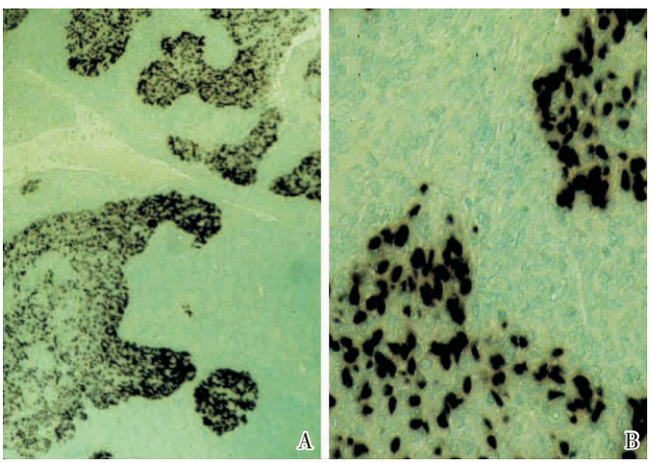

Figure 9 Locoligation of EBV signal in NPC biopsy specimen by EBER-1 in situ hybridization

A and B: Detection of EBV small nuclear RNA by digoxigenin-labeled EBER-1 anti-sense oligonucleotide probe. A: EBV-containing tumor cells are seen in patches similar to a geographic map pattern within a tumor nest (×25). B: At higher magnification from another biopsy section, EBV-containing tumor cells are intermingled with EBV$^-$ tumor cells. A few EBV$^+$ lymphocytes are also shown (×100).

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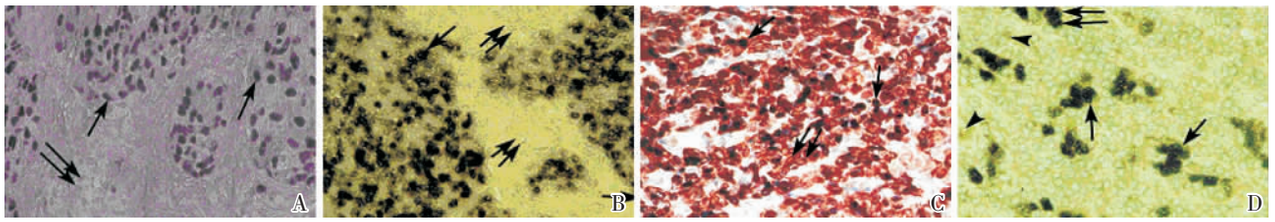

Figure 10 Localization of EBER-1 and EBV DNA, double localization of cytokeratin and EBER-1, and double localization of IgAR and EBER-1 in NPC biopsy specimens

A: EBER-1 in situ hybridization in one NPC specimen shows several tumor nests containing EBV$^+$ (single arrows) and EBV$^-$ tumor cells. The other tumor nest (double arrows) shows EBV$^-$ tumor cells only. B: In situ PCR hybridization of other NPC biopsy specimens shows geographical distribution of EBV+ tumor cells (single arrow) in the peripheral areas and EBV$^-$ tumor cells (double arrows) in the central areas. C: Double localization of cytokeratin and EBER-1 in one NPC biopsy specimen. The deep brown reaction product representing the cytokeratin is shown in all NPC cells. But only a fraction of those tumor cells contains black reaction product of EBER-1 signal (arrows). In between the tumor cells are lymphocytic and stromal cells (counter stained with week hematoxylin). D: Double localization of EBER-1 and IgAR (secretory component protein) in the NPC specimens. Some EBV$^+$ tumor cells (black stained nuclei) contain brownish yellow color of IgAR protein (single arrows). Other tumor cells contain only EBV signal with very weak or no IgAR protein (double arrows). Another tumor cells only express IgAR (arrowheads), while one fraction of tumor cells contains neither IgAR nor EBV signal. (A: EBER-1 in situ hybridization; B: in situ PCR hybridization; C: EBER-1 in situ and IHC staining of CK; D: EBER-1 in situ and IHC staining of SC protein, A–D: ×100)

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squamous cells in a normal thymus also express IgAR (our unpublished data). The expression of the SC protein in keratinized tumor cells may explain the finding that in some acquired immune deficiency syndrome (AIDS) patients, their oral mucosae especially relative to the tongue may exhibit some hairy leukoplakia lesions, which exhibit EBV signals in the superficial half layer of keratinized epithelia, but not in the basal half layer of epithelia.[57]

2.3 Co-existence of EBV and IgAR in NPC biopsy specimens

In most NPC biopsy specimens, if double localization of EBER-1 and IgAR is performed, four types of NPC cells can usually be identified: tumor cells with IgAR$^-$, EBV$^-$; IgAR$^+$, EBV$^+$; IgAR$^+$, EBV$^-$; and IgAR$^-$, EBV$^+$ (Figs. 10D and 12C).[34] Tumor cells that do not express IgAR cannot be infected by EBV. But even the cells expressing IgAR may be also free of EBV infection if the extracellular space has (1) no free EBV particles, or (2) no IgA anti-EBV-VCA, or (3) neither free EBV particles nor IgA anti-EBV-VCA. The presence of EBV signals and IgAR in the same tumor cells indicates that EBV has infected these cells through IgAR (Figs. 10D and 12C). The tumor cells with IgAR$^+$ and EBV$^+$ may become dominant in advanced cases, in which most tumor cells contain EBV signals and very weak or even invisible IgAR (Fig. 12D).[34] It stems from the fact that some of the EBV protein suppress the expression of IgAR gene in infected host cells (Fig. 13).[36] These phenomena indicate that the transformed tumor cells cannot be infected by EBV unless they express

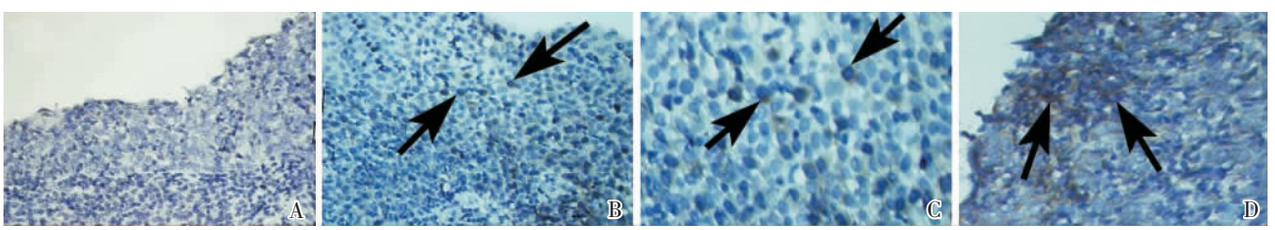

Figure 11 Immunolocalization of IgAR in NPC biopsy specimens

A: NPC in situ with focal invasion shows no IgAR staining in any single tumor cell. B: An early invasive NPC shows only a few tumor cells containing IgAR immunoreactivity (arrows). C: Higher magnification from B. The IgAR-containing tumor cells are shown (arrows). D: Another carcinoma in situ with early invasion shows more tumor cells stained by IgAR reaction product (arrows). (A–D: IHC staining of SC protein, counter stained with regular hematoxylin; A, B: ×50; C, D: ×100).

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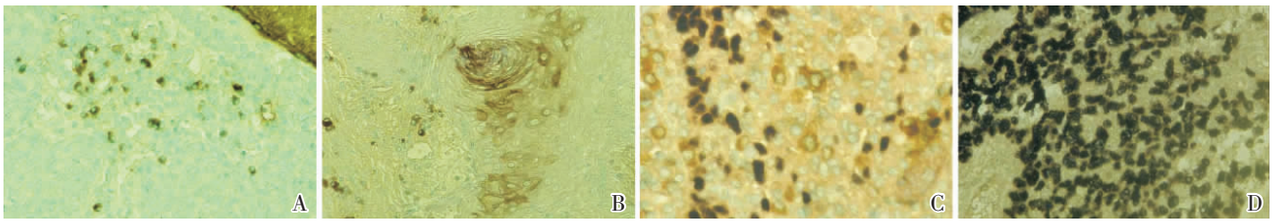

Figure 12 Localization of SC protein in original NPC biopsy specimens

A: A type Ⅲ NPC specimen. The reaction product of anti-SC is shown in the cytoplasm and plasma membrane of certain tumor cells only. The majority of tumor cells are unstained. The covering squamous metaplastic epithelia are not stained. The right upper corner with strong staining is due to the blood clot, which contains endogenous peroxidase activity of hemoglobin. B: A type Ⅰ NPC specimen. The keratinizing squamous carcinoma cells show specific immunostaining. Some non-keratinizing NPC tumor cells in other areas also show a reaction product. C, D: Double localization of EBER-1 and IgAR (secretory component protein) in the NPC specimens. C: Some EBV+ tumor cells (black-stained nuclei) contain brownish yellow color of IgAR protein. D: Other tumor cells contain only EBV signal with very weak or no IgAR protein. Another tumor cells only express IgAR, while one fraction of tumor cells contains neither IgAR nor EBV signal. (A, B: IHC localization of SC protein; C, D: Double localization of EBER-1 in situ and IHC staining of SC protein, A, B: ×50; C, D: ×100).

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IgAR. Once the tumor cells are infected by EBV, their proliferation becomes faster than that of surrounding EBV$^-$ cells, resulting in clonal expansion and formation of the geographic distribution of EBV$^+$ cells in the originally EBV$^-$ tumor nests or nodules. Then, the expression of IgAR in EBV$^+$ NPC cells gradually decreases; thus, EBV$^+$ NPC cells can prevent secondary infection from other EBV particles.

2.4 Molecular regulation of NPC host gene expression by EBV-encoded latent membrane protein 1 (LMP1)

EBV LMP1 is known as an oncogene and a transforming protein because it can transform immortalized rodent fibroblasts,[58] but cannot transform the fibroblasts in primary cultures (our unpublished data) and is essential for EBV-induced B-cell transformation (immortalization) in vitro.[54,55,58,59] LMP1 functions as a constitutively activated member of the tumor necrosis factor receptor (TNFR) super family, and activates several signaling pathways in a ligand-independent manner.[58-63] LMP1 protein activates several downstream signaling pathways that contribute to many phenotypic consequences of LMP1 expression, including the induction of various genes that encode anti-apoptotic proteins and cytokines.[63,64]

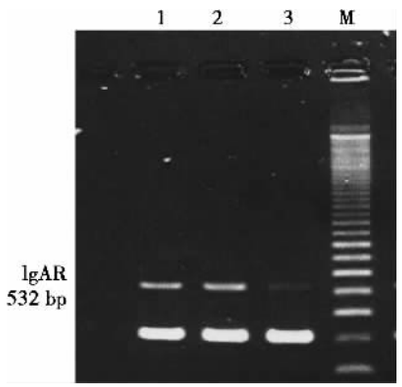

Figure 13 Semi-quantitative RT-PCR of SC mRNA expression in NPC cells

Total RNA were extracted from EBV$^+$ and EBV$^-$ NPC cells for RT-PCR analysis. Lane 1: EBV$^-$ cells; lane 2: NPC cells infected with EBV for one day; lane 3: from NPC cells infected with EBV for seven days; lane M: marker. Marked decrease in SC mRNA expression is shown in NPC cells infected with EBV for 7 days (lane 3).

In EBV$^+$ xenografts in SCID mice, a fraction of EBV$^+$ cells expresses EGFR intensively while the remaining fraction of EBV$^+$ cells expresses no EGFR; however, in EBV$^-$ xenografts, a fraction of EBV$^-$ tumor cells expresses EGFR protein moderately while the remaining also shows no EGFR expression (Fig. 8E-F).[36] These findings indicate that EBV can enhance EGFR gene expression in EGFR-positive tumor cells, but cannot turn on EGFR gene expression in EGFR-negative tumor cells.[36] This phenomenon can also be demonstrated in an in vitro experiment. In an NPC cell line (TW-06) with heterozygous p53 point mutation,[65] when pmdm2 promoter-luciferase (pmdm2-Luc) and pCMV-LMP-1 are co-transfected, no mdm2-Luc activity is observable, but if pmdm2-Luc and pSV40-p53 are co-transfected, the mdm2-Luc activity increases moderately (by about 2.5 folds); however, when pmdm2-Luc, pSV40-p53, and pCMV-LMP-1 are co-transfected, the mdm2-Luc activity is highly up-regulated by 6.3 folds. This finding substantiates a hypothesis obtained from the EBV$^+$ xenograft experiment: in EBV$^+$ xenografts, EBV LMP1 can enhance the expression attributable to a host gene (mdm2) in cells that already express mdm2, but cannot turn on the expression of mdm2 in cells that do not express mdm2.[53] In other words, EBV plays a role as an enhancer to enhance the progression of NPC tumorigenesis and is unlikely to

play a role as an initiator or a promoter.

2.5 The role of EBV in the pathogenesis of NPC

In NPC biopsy specimens, which may contain areas of squamous metaplasia, mild dysplasia, severe dysplasia, CIS and invasive carcinoma in the nasopharyngeal mucosa, EBV signals are observable only in certain transformed epithelial cells (involving severe dysplasia or CIS and invasive carcinoma) but not in the untransformed squamous metaplastic epithelia or mild dysplasia.[37] Our recent intensive investigation of 209 NPC biopsy specimens supported this finding.[56] Similar findings have also been reported in previous literature,[32,66] as mentioned above. Unfortunately, this finding cannot support a claim from a very unusual summary in Pathology and Genetics of Head and Neck Tumors that "EBV latent infection appears when low-glade dysplasia progresses to high-grade dysplasia and the near constant association of EBV with NPC irrespective of ethnic background." To challenge this unusual statement, we have performed another experiment examining EBV signals in tissues that have CIS and present evidence that CIS (severe dysplasia) exhibits EBV signals but that the mild dysplasia of NPC biopsy specimens does not exhibit EBV signals. A few cases of CIS (severe dysplasia) and early invasive cases exhibited sporadic EBV$^+$-transformed cells (Figs. 14 and 15A-B),[54-56] and rare cases (only in one out of 209 cases) exhibited many EBV$^+$-transformed cells (Figs. 15 C-D).[56] However, most cases exhibited no EBV infection. Instead, the most frequently identified epithelial carcinoma cells containing EBV signals were in NPC tumor nodules (Figs. 9, 10 and 12 C-D).[31,56] In other words, EBV cannot infect (1) normal oral squamous epithelial cells, (2)nasopharyngeal squamous metaplastic cells, or (3) mild dysplasia, mainly because these three categories of cells express neither the EBV receptor (CD21) nor the IgA receptor (SC protein).[56] These facts strongly support the hypothesis that EBV could not infect normal oro-nasopharyngomucosal epithelia, and that EBV can not use these sites as niches for further proliferation, for dissemination, and for the transformation of those epithelial cells.

Furthermore, histopathologic

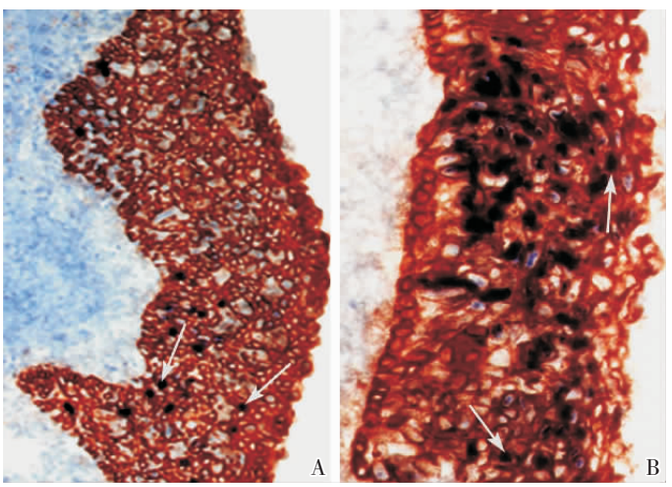

Figure 14 Double localization of cytokeratin and EBER-1 in NPC in situ in two biopsy specimens

A: Carcinoma in situ with early invasion shows only a few tumor cells containing EBV signal (single arrow). B: Another NPC biopsy specimen shows carcinoma in situ containing more EBV$^+$ tumor cells (arrows). Dark brown reaction product: cytokeratin; black stained area: EBER-1 signal. (A, B: EBER-1 in situ and IHC staining of cytokuatin, counter stained with weak hematoxylin; A: ×50; B: ×100).

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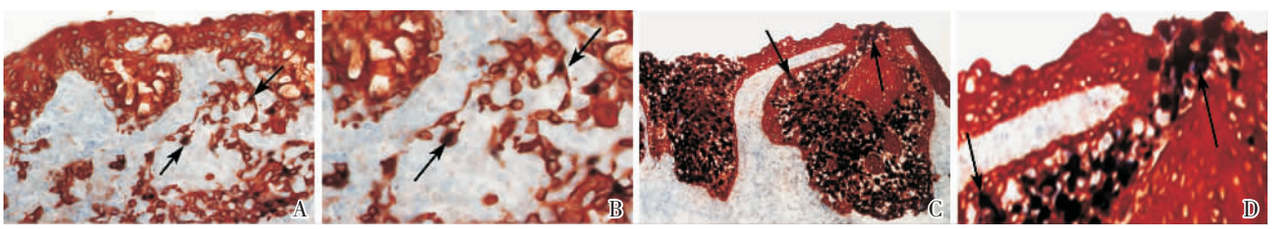

Figure 15 Double localization of cytokeratin and EBER-1 by immunohistochemistry and EBER-1 in situ hybridization in NPC biopsy specimens

A: Carcinoma in situ with early invasion shows only a few tumor cells in the submucosal invasive region containing EBV signals (arrows). B: Higher magnification from A (arrows). C: An early invasive carcinoma shows numerous tumor cells containing EBV signals (arrows). But some EBV- tumor cells are still visible. D: Higher magnification from C (arrows). (A-D: EBER-1 in situ and IHC staining of cytokeratin, counter stained with weak hematoxylin; A, C: ×50; C, D: ×100).

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examination of oral mucosa relative to infectious mononucleosis patients has never revealed the presence of EBV signals in squamous cells although the mucosae are heavily infiltrated with EBV^+ lymphocytes.[67] This finding is inconsistent with another hypothesis that EBV can get into the squamous epithelial cells by the fusion of epithelial cells with EBV^+ lymphocytes. In this same regard, we should keep in mind two important facts: (1) certain transformed epithelial cells (NPC) can express SC protein on the cell membranes; and (2) both SC protein expression and EBV signals are identifiable in the same NPC cells. These two facts strongly indicate that EBV can infect NPC cells through IgAR-mediated endocytosis if both free EBV particles and IgA anti-EBV-VCA are present in the interstitial fluid in the tumor tissue, as mentioned above.

There are no EBV signals in most cases of CIS and early invasive NPC, except for a few cases involving EBV signals in a few transformed cells (Figs. 14A and 15A-B) and for a few other particular cases (Figs. 14B and 15C-D); similarly, only a few transformed cells may express IgAR (Fig. 11).[34,56] In other words, identification of EBV signals in transformed cells in CIS is not a common phenomenon.

It is a general contention that the definitive designation of a lesion as "EBV-associated" should require unequivocal demonstration of the presence of the EBV genome or viral gene product in most tumor cells.[55] Latent infection and IgAR have not been detected in normal squamous metaplastic epithelial cells,[31,34,65] and the examples of latent EBV-infected epithelial cancer cells concern only a few cases of CIS and invasive NPC cells,[56] as mentioned above; in addition, EBV^+ CIS is not a common phenomenon, and some NPC cells in the tumor masses always exhibit

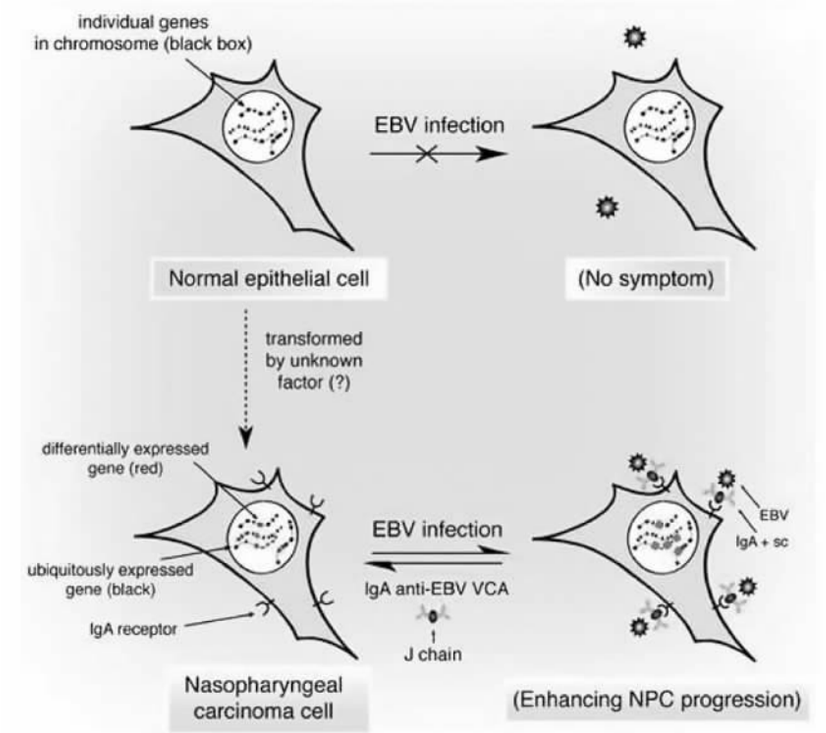

Figure 16 Hypothetical model for the effect of EBV on NPC cells in the light of statistical evidence using genome-wide microarray screening

EBV causes host gene alteration only under a situation where a normal squamous epithelial cell was transformed (such as NPC in this case) and there is expression of the IgA receptor because normal squamous epithelial cells have no receptor for EBV infection. The gene expression regulated by EBV enhances the NPC gene expression profile by modifying the already differentially expressed genes and keeping the ubiquitously expressed genes untouched. The small red boxes (left lower corner) indicate the differentially expressed genes (including up and down regulated genes) and where the statistically significant majority were affected and amplified as denoted in bigger red boxes (right lower corner). It is worth noting that the reversible event of EBV infection was frequently observed in vitro, but has seldom been seen in vivo.

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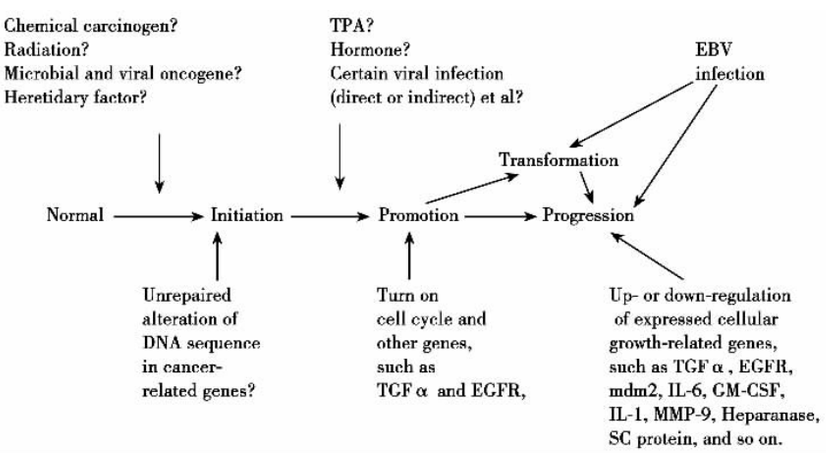

Figure 17 Schematic presentation of the role of EBV in NPC pathogenesis

no EBV signals. These findings all suggest that early transformed cells are not necessarily infected by EBV; instead, they can proliferate by themselves. During the proliferation of early transformed cells, some additional genetic changes may occur. If IgAR is expressed by some of these proliferated transformed cells, then they can be infected by EBV, regardless of whether they are at the early transformed stage or at the tumor progression stage; clonal expansion of those EBV-infected cells may occur. Figure 16 schematically presents a model of both EBV infection and genetic alteration in epithelial cancer cells, and Figure 17 schematically presents the role of EBV in the pathogenesis of NPC.[51,56] Therefore, it is reasonable to state that in the processes of NPC carcinogenesis, EBV is an "accessory to the crime," and not the "primary culprit."

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